STABLE RECOMBINANT YEASTS
FOR FERMENTING XYLOSE TO ETHANOL

BACKGROUND

The present invention relates generally to genetically engineered microorganisms and in particular to unique methods for stably incorporating exogenous DNA into cells, including the incorporation of multiple copies of the exogenous DNA at reiterated DNA sequences in the host. In a preferred aspect, the invention relates to yeasts capable of fermenting xylose (preferably cofermenting the same with glucose) to ethanol. More particularly, a preferred aspect of the invention relates to yeasts containing cloned genes encoding xylose reductase (XR), xylitol dehydrogenase (XD), and xylulokinase (XK), which yeasts substantially retain their efficiency for fermenting xylose to ethanol even after culturing in non-selective medium for a large number of generations.

As further background, recent studies have proven ethanol to be an ideal liquid fuel for automobiles. It can be used directly as a neat fuel (100% ethanol) or as a blend with gasoline at various concentrations. The use of ethanol to supplement or replace gasoline can reduce the dependency of many nations on imported foreign oil and also provide a renewable fuel for transportation. Furthermore, ethanol has proven to provide cleaner fuels that release far fewer pollutants into the environment than regular gasoline. For example, it has been demonstrated that the use of oxygenated materials in gasoline can reduce the emission of carbon monoxide, a

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harmful pollutant, into the air. Among the several
oxygenates currently used for boosting the oxygen content
of gasoline, ethanol has the highest oxygen content. The
United States Environmental Protection Agency (EPA) has
5   shown that gasoline blended with 10% ethanol reduces
carbon monoxide emissions by about 25% - 30%.

Up to now, the feedstock used for the production of
industrial alcohol by fermentation has been sugars from
10   sugar cane or beets and starch from corn or other food
crops. However, these agricultural crops are presently
considered to be too expensive to be used as feedstock
for the large-scale production of fuel ethanol. Plant
biomass is an attractive feedstock for ethanol-fuel
15   production by fermentation because it is renewable, and
available at low costs and in large amounts. The concept
of using alcohol produced by microbial fermentation of
sugars from agricultural biomass had its nascence at
least two decades ago. The major fermentable sugars from
20   cellulosic materials are glucose and xylose, with the
ratio of glucose to xylose being approximately 2 or 3 to
1. The most desirable fermentations of cellulosic
materials would, of course, completely convert both
glucose and xylose to ethanol. Unfortunately, even now
25   there is not a single known natural microorganism capable
of fermenting both glucose and xylose effectively.

Yeasts, particularly *Saccharomyces* yeasts, have
traditionally been used for fermenting glucose-based
30   feedstocks to ethanol, and they are still considered the
best microorganisms for that purpose. However, these
glucose-fermenting yeasts, including the *Saccharomyces*

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yeasts, have been found to be unable to ferment xylose
and also unable to use this pentose sugar for growth.

Recently, N. Ho et al. have developed recombinant
yeasts, particularly recombinant *Saccharomyces* yeasts,
capable of effectively fermenting xylose to ethanol (Ho
and Tsao, 1995). More particularly, the preferred
recombinant yeasts were capable of co-fermenting the two
major sugar constituents of cellulosic biomass, glucose
and xylose, to ethanol (Ho and Tsao, 1995). These
recombinant yeasts were developed by the transformation
of yeasts with a high-copy number plasmid containing
three cloned genes, XR, XD, and XK, encoding three key
enzymes for xylose metabolism (Figure 1). Figure 2 and
Figure 3 demonstrate two of the prior-made recombinant
Saccharomyces yeasts, designated 1400 (pLNH32) and
1400(pLNH33), capable of co-fermenting 8% glucose and 4%
xylose present in the same medium almost completely to
ethanol in two days. On the other hand, Figure 4 shows
that the parent yeast fusion 1400 (D'Amore, et al., 1989
and D'Amore, et al., 1990) can only ferment glucose, but
not xylose, to ethanol. 1400 (pLNH32) (in short LNH32)
and 1400(pLNH33) (in short LNH33) were developed by the
transformation of the *Saccharomyces* fusion 1400 (D'
Amore, et al., 1989 and D'Amore, et al., 1990) with two
of the high-copy-number plasmids, pLNH32 and pLNH33,
shown in Figure 1. To date, there have been four such
high-copy-number plasmids reported, pLNH31, pLNH32,
pLNH33, and pLNH34 (Ho and Tsao, 1995). Each of these
plasmids can transform fusion 1400 to recombinant yeasts
to co-ferment both glucose and xylose with similar
efficiencies.

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Yeasts 1400 (pLNH32), 1400(pLNH33), and related
recombinant xylose-fermenting *Saccharomyces*, with their
xylose metabolizing genes cloned on a 2µ-based stable

5   high-copy-number plasmid, are quite suitable for a batch
process fermentation. However, in a continuous process
fermentation, after prolonged culture in a glucose-rich
medium (more than 20 generations), 1400 (pLNH32),
1400(pLNH33), and similar plasmid-mediated recombinant

10   yeasts lose their capability of fermenting xylose as
shown in Figure 5 and Figure 6.

Generally, exogenous DNA or gene(s) can be cloned
into yeasts by two separate ways. One way is to clone

15   the exogenous DNA or gene(s) into a plasmid vector
containing a selectable genetic marker and a functional
yeast DNA replication origin or ARS (autonomous
replicating sequence) (Struhl et al., 1979; Stinchcomb et
al., 1980; Chan and Tye, 1980) that allows the plasmid to

20   be able to replicate autonomously in its new host,
followed by transformation of the desired yeast host with
the plasmid containing the cloned DNA fragment or
gene(s). The resulting yeast transformants are able to
stably maintain the cloned gene in the presence of

25   selection pressure. However, such cloned gene(s) are
unstable after prolonged culture in non-selective medium
(in the absence of selection pressure).

Another way to clone the exogenous DNA or gene(s)

30   into a yeast host is to integrate the DNA or gene(s) into
the yeast chromosome. In yeast, integrative
transformation is almost always via homologous

recombination (Orr-Weaver, 1981). The simplest way to
clone a desired gene into a yeast chromosome by
integration is first to clone the desired gene into a
plasmid which does not contain a replication of origin or

5   ARS (autonomous replication sequences) but does contain a
piece of the host DNA for targeting the integration to a
specific site (Orr-Weaver, 1981). Transformation of the
new yeast host with such an intact integrative vector
will generate integrative transformants containing the

10   desired gene cloned to the site next to the selected
targeting yeast DNA sequences. However, the frequency of
such integrative transformation is extremely low (1 to 10
transformants per μg DNA). Subsequently, it has been
demonstrated that integrative vectors linearized within

15   the DNA fragment homologous to the host chromosomal DNA
can transform yeasts with much higher frequencies (100-
to 1000-fold higher) (Orr-Weaver, 1981; Orr-Weaver and
Szostak, 1983). It was suggested that double-stranded
breaks, introduced by restriction enzyme digestion, are

20   recombinogenic and highly interactive with homologous
chromosomal DNA. This is particularly helpful for a
complex plasmid, containing more than one yeast gene, so
that one can direct the integration to a specific site by
making a restriction enzyme cut within the corresponding

25   region on the plasmid.

Another type of integration, also described as
transplacment or gene disruption, makes use of double
homologous recombination to replace yeast chromosomal DNA

30   (Rothstein, 1981). Double homologous recombination
vectors contain the exogenous DNA or gene(s) to be cloned
and the selection marker, flanked by yeast DNA sequences

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homologous to 5' and 3' regions of the segment of
chromosomal DNA to be replaced. Prior to transformation,
the vector is digested with restriction enzymes which
liberate the transplacing fragment containing 5' and 3'
5   ends homologous to the chromosomal DNA sequences at the
desired integration sites. The latter strategy has
become the method of choice for integrative
transformation of yeast if a stable single-copy
transformant is desired.

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A number of strategies based on integration into
reiterated chromosomal DNA have been used to generate
stable multiple-copy integrants. For example, the delta
sequence of yeast retrotransposon Ty (Sakai et al., 1990;
15   Sakai et al., 1991), the highly conserved repeated sigma
element (Kudla and Nicolas, 1992) and non-transcribed
sequences of ribosomal DNA (Lopes et al., 1989; Lopes et
al., 1991; Rossolini et al., 1992) have all been used as
the target sites for multiple integration of exogenous
20   gene(s) into yeast (Rothstein, 1991; Romanos et al.,
1992).

Recent work reported in the literature on multiple
integration of exogenous genes into the yeast chromosome
25   has for the most part involved the use of either properly
linearized non-replicative vectors or DNA fragments
containing the desired gene(s) to be cloned and the
genetic marker for selection, flanked with DNA sequences
homologous to a region of yeast chromosomal DNA. Rarely,
30   linearized replicative vectors and almost never intact
replicative vectors, such as intact ARS vectors, were
used to achieve such recombinant transformation. Thus,

since early work at the onset of developing yeast
integrative transformation, (Szoatak and Wu (1979)), and
despite the observation that DNA cloned on ARS vectors
can integrate into the host chromosomes (Cregg et al.,
5   1985; Kurtz et al., 1986), the use of intact ARS vectors
(Struhl et al., 1979; Stinchcomb et al., 1980; Chan and
Tye, 1980) for integration purposes has long since
generally been abandoned. This has especially been true
since the discovery that the double-stranded breaks
10   introduced by restriction enzyme digestion are
recombinogenic (Orr-Weaver, 1981; Orr-Weaver and Szostak,
1983).

In light of this background, there remain needs for
15   more stable yeast which ferment xylose to ethanol,
preferably xylose and glucose simultaneously to ethanol,
and for facile and effective methods for making high copy
number integrants. The present invention addresses these
needs.

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SUMMARY OF THE INVENTION

Accordingly, the present invention provides yeast
containing multiple copies of stably cloned XR, XD, and
XK genes, which even upon culture in non-selective medium
for multiple generations (e.g. greater than 20) retain
their full capability to ferment xylose to ethanol. More
preferably, the XR, XD, and XK genes are all fused to
promoters not inhibited by the presence of glucose and
also not requiring the presence of xylose for their
expression. Still more preferably, the yeast of the
invention can co-ferment the two major constituents of
cellulosic biomass, glucose and xylose, to ethanol.

Another embodiment of the present invention relates
to the use of reiterated sequences, e.g. non-transcribed
r-DNA sequences adjacent to the 5S DNA (Valenzuela et
al., 1977), as homologous sequences for targeting high-
copy-number integration of a DNA fragment containing XR,
XD, and XK into the yeast genome via homologous
recombination. For example, a replicative plasmid vector
including the DNA fragment flanked by the homologous
sequences can be used to target integration of the DNA
fragment. A preferred method of the invention includes
the steps of (a) transforming the cells with a
replicative/integrative plasmid having exogenous DNA
including a selection marker; and (b) repeatedly
replicating the cells from step (a) to produce a number
of generations of progeny cells while selecting for cells
which include the selection marker (e.g. by replicating
on selective plates), so as to promote the retention of
the replicative and integrative plasmid in subsequent

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generations of the progeny cells and the formation of
progeny cells having multiple integrated copies of the
exogenous DNA. In a further step, the cells from step
(b) can be replicated to produce a number of generations
5   of progeny cells in the absence of selection for cells
which include the selection marker, so as to promote the
loss of the plasmid in subsequent generations of progeny
cells (thus leaving an enriched population of the stable
integrants).

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The invention also provides an advantageous mode for
selection and maintenance of the desired transformants.
It is well known that in minimal medium all
microorganisms require the presence of a carbon source,
15   such as glucose or xylose, for growth. However, most
microorganisms do not require the presence of a carbon
source for growth in rich medium. Nevertheless, the
present invention provides the use a carbon source as the
selection pressure for the selection of transformants
20   even in rich medium, such as YEP (1% yeast extract plus
2% peptone). The development of stable transformants,
such as 1400(LNH-ST) (Figure 7), which are capable of
effective fermentation of xylose after culturing in non-
selective medium for essentially unlimited generations,
25   has been greatly facilitated by the discovery that many
yeasts, particularly *Saccharomyces* yeasts, do naturally
require the presence of a carbon source, such as xylose
or glucose, for growth even in rich medium, as shown in
Figure 8.

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In a broad aspect, the invention also provides a
method for integrating multiple copies of exogenous DNA

into reiterated chromosomal DNA of cells. The method includes (a) transforming the cells with a replicative and integrative plasmid having exogenous DNA including a selection marker. The method also includes (b)

5 replicating the cells from step (a) to produce a number of generations of progeny cells while selecting for cells which include the selection marker, so as to promote the retention of the replicative and integrative plasmid in subsequent generations of the progeny cells and produce

10 progeny cells having multiple integrated copies of the exogenous DNA. In a specific application, such a method includes (i) transforming yeast cells with a replicative plasmid having exogenous DNA including a selection marker, the exogenous DNA being flanked on each end by a

15 DNA sequence homologous to a reiterated sequence of DNA of the host; (ii) repeatedly replicating the transformed yeast cells from step (i) to produce a number of generations of progeny cells while selecting for cells which include the selection marker, so as to promote the

20 retention of the replicative plasmid in subsequent generations of the progeny cells and result in progeny cells each containing multiple integrated copies of the exogenous DNA; and (iii) replicating the progeny cells from step (ii) to produce a number of generations of

25 progeny cells in the absence of selection for cells which include the selection marker, so as to promote the loss of the plasmid in subsequent generations of progeny cells and recover yeast cells each containing multiple copies of the exogenous DNA integrated into its chromosomal DNA.

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    In still another embodiment, the invention provides a yeast which ferments xylose to ethanol, the yeast

having multiple copies of exogenous DNA integrated into
its chromosomal DNA. The exogenous DNA including genes
encoding xylose reductase, xylitol dehydrogenase, and
xylulokinase fused to non-glucose-inhibited promoters,
5 wherein the yeast ferments glucose and xylose
simultaneously to ethanol and substantially retains its
capacity for fermenting xylose to ethanol for at least 20
generations even when cultured under non-selective
conditions.

10   Another aspect of the invention relates to methods
for fermenting xylose to ethanol, which include
fermenting xylose-containing mediums with yeasts of the
invention.

15   Another embodiment of the invention provides a
plasmid vector for integrating an exogenous DNA sequence
including a selection marker into chromosomal DNA of a
target yeast cell. The inventive plasmid vector contains
20 a functional yeast DNA replication origin and the
exogenous DNA including the selection marker flanked on
each end by a DNA flanking sequence which is homologous
to a reiterated ribosomal DNA sequence of the target
yeast cell. The plasmid further has a second selection
25 marker in a position other than between the DNA flanking
sequences.

A still further embodiment of the invention provides
a plasmid vector for integrating an exogenous DNA
30 sequence into a yeast to form stable integrants which
ferment xylose to ethanol. The vector contains a
functional yeast DNA replication origin and exogenous DNA

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including genes encoding xylose reductase, xylitol
dehydrogenase, and xylulokinase flanked on each end by a
DNA flanking sequence which is homologous to a reiterated
DNA sequence of the target yeast cell.

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        A still further aspect of the invention provides a
method for forming cells having multiple integrated
copies of an exogenous DNA fragment. This inventive
method includes replicating cells having reiterated

10   genomic DNA and which contain a replicative and
integrative plasmid containing the exogenous DNA to
produce multiple generations of progeny cells while
selecting for cells which include the selection marker,
so as to promote the retention of the replicative and

15   integrative plasmid in subsequent generations of the
progeny cells and produce progeny cells having multiple
integrated copies of the exogenous DNA.

        The invention provides yeasts containing stably

20   cloned genes enabling their use under non-selective
conditions (e.g. continuous fermentations) to coferment
xylose and glucose to ethanol, while not losing their
capacity to ferment xylose. In addition, the invention
provides methods and materials for forming stable,

25   multiple-copy integrants of yeast and other cells which
are facile to perform and which can be controlled to
modulate the number of copies of the integrated exogenous
DNA. Additional embodiments, and features and advantages
of the invention will be apparent from the following

30   description and appended claims.

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BRIEF DESCRIPTION OF THE FIGURES

        Figure 1 shows the restriction map of the plasmids
pLNH31, -32, -33, and -34, and the genes cloned within.

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        Figure 2 shows that yeast transformant 1400(pLNH32)
(in short LNH32) can effectively coferment glucose and
xylose. The conditions used for culturing the yeast and
for fermenting the sugars are similar to those described

10   in Example 7.

        Figure 3 shows that yeast transformant 1400 (pLNH33)
(in short LNH33) can effectively coferment glucose and
xylose. The conditions used for culturing the yeast and

15   for fermenting the sugars are similar to those described
in Example 7.

        Figure 4 shows that the parent yeast fusion strain
1400 can ferment glucose but not xylose. The conditions

20   used for culturing the yeast and for fermenting the
sugars are similar to those described in Example 7.

        Figure 5 demonstrates that yeast transformant
1400(pLNH32) (in short LNH32) with its xylose

25   metabolizing genes cloned in the replicative plasmid
pLNH 32 is not stable in a non-selective medium. After
being cultured for 20 generations in a non-selective (for
example, glucose) medium, 1400(pLNH32) lost its
capability to ferment xylose.

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Figure 6 demonstrates that yeast transformant
1400(pLNH33) (in short LNH33) with its xylose
metabolizing genes cloned in the replicative plasmid
pLNH 33 is not stable in a non-selective medium. After
5 being cultured for 20 generations in a non-selective
medium (for example, glucose medium), 1400(pLNH33) lost
its capability to ferment xylose.

Figure 7 shows that yeast transformant 1400(LNH-ST)
10 (in short LNH-ST) can stably maintain its xylose
fermenting capability even after being cultured in non-
selective medium for more than 40 generations.

Figure 8 demonstrates that *S. cerevisiae* and other
15 *Saccharomyces* yeasts require a carbon source for growth
even when rich media such as yeast extract and pepton
were present in the medium. For example, these
experiments showed that *S. cerevisiae* was unable to
grow in the YEP medium containing 1% yeast extract and 2
20 % pepton, but was able to grow when glucose or xylulose
was added to the YEP medium.

Figure 9A shows the restriction map of pLNH-ST, and
the genes cloned within.
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Figure 9B shows the genetic map (the order and
orientation) of genes (5S rDNA, KK, AR, and KD) cloned in
pLNH-ST. The oligonucleotides (for example, Oligo 25,
Oligo 26, etc.) that are above or below the gene map are
30 the primers used to characterize the order and
orientation of the cloned genes by PCR.

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Figure 10 is a schematic diagram outlining the construction of pBluescript II KS(-) containing the cloned XR, XD, XK genes: four such plasmids were constructed. The KK-AR-KD fragment cloned in pKS(-)-KK-

5   AR-KD-3 was chosen to be cloned in pUCKm-rDNA(5S)-ARS for the construction of pUCKm-rDNA(5S)(KRD)-ARS, also designated as pLNH-ST.

Figure 11 shows that yeast transformant 1400(LNH-ST)

10   (in short LNH-ST), superior to 1400(pLNH 32) and 1400(pLNH 33), can effectively coferment glucose and xylose. The conditions used for culturing the yeast and for fermenting the sugars are similar to those described in Example 7.

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Figure 12 shows the genes cloned in and the restriction map of a broad-host plasmid for the isolation of ARS containing DNA fragments from the chromosome DNA of *S. cerevisiae* and other yeasts.

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DETAILED DESCRIPTION OF THE INVENTION

For the purpose of promoting an understanding of the
principles of the invention, reference will now be made to
5   certain preferred embodiments thereof, and specific language
will be used to describe the same. It will nevertheless be
understood that no limitation of the scope of the invention
is thereby intended, such alterations, further modifications
and applications of the principles of the invention as
10 described herein are being contemplated as would normally
occur to one skilled in the art to which the invention
relates.

As mentioned above, one preferred aspect of the
15 present invention provides recombinant yeasts
incorporating stably cloned XR, XD and XK genes, which
represent an improvement upon prior-reported recombinant
yeasts. Generally, recombinant yeasts that can
effectively coferment both glucose and xylose present in
20 the same medium have been reported (Ho and Tsao, 1995).
The yeasts made in this publication were accomplished by
cloning properly modified XR, XD, and XK genes on a high
copy number plasmid, pUCKm10, followed by using the
resulting plasmid, pLNH3X (X=1 to 4) (Figure 1), to
25 transform suitable natural yeasts. For example, the
plasmids pLNH32 and pLNH33 were used to transform fusion
yeast 1400 to 1400 (pLNH32) and 1400 (pLNH33),
respectively. These recombinant _Saccharomyces_ yeasts can
effectively coferment both glucose and xylose present in
30 the same medium to ethanol as shown in Figures 2 and 3,
while the parent unengineered 1400 yeast can only ferment

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glucose alone, not coferment both glucose and xylose
(Figure 4).

Plasmid-mediated recombinant yeasts can maintain the
cloned genes in the presence of selection pressure, but
not in the absence of selection pressure. As
demonstrated in Figures 5 and 6, 1400 (pLNH32) and 1400
(pLNH33) eventually lose their plasmids and their
capability for fermenting xylose after prolonged culture
in the absence of selection pressure.

It is highly desirable that recombinant industrial
yeasts, particularly those strains used for the
production of large volume industrial products, such as
ethanol, be stable without requiring the presence of
selection. The development of recombinant yeasts
containing integrated XR, XD, and XK genes, as in the
present invention, provides such stability. In addition,
for the resulting recombinant yeasts to have the ability
to coferment glucose and xylose at efficiencies similar
to or better than 1400(pLNH32) and 1400(pLNH33), the
recombinant yeasts must contain not only the integrated
xylose metabolism genes, but also high numbers of copies
of such integrated genes. In preferred aspects of the
present invention, high-copy-number (hcn) integrants of
yeasts (i.e. yeasts having at least about 10 integrated
copies of the exogenous DNA) have been developed by
targeting a non-coding region, such as a non-coding
region of 5S ribosomal DNA (rDNA) as the site for
multiple integration.

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rDNA provides an advantageous location for integration because it is highly conserved, and yeasts generally contain more than 100 copies of the rDNA repeated sequences. It will be understood, however, that
5   to achieve yeasts of the present invention, it will not be necessary to achieve integration of the desired genes at every occurrence of a repeated or reiterated sequence. It will be sufficient to achieve such integration at each of multiple sites of a reiterated sequence, i.e. two or
10   more sites, in accordance with the broad aspects of the present invention.

In order to integrate hcn XR, XD, and XK into the yeast chromosome at the site of 5S rDNA, the integration
15   plasmid, pLNH-ST, as shown in Figure 9, was constructed. pLNH-ST is a yeast-*E. coli* shuttle vector and a derivative of pUCKm 6 plasmid (Ho et al., 1984). The 5S rDNA sequences was inserted at the Xho I restriction site of pUCKm 6. The 5S rDNA sequence was copied from the
20   yeast chromosomal DNA by the PCR technique and modified by the site-specific mutagenesis technique to add an XhoI restriction site in its center (approximately) sequence as shown in Figure 9. The XhoI fragment from pKS(-)-KK-AR-KD (Figure 10) (Ho and Tsao, 1995) has been inserted
25   into the XhoI site of the 5S rDNA cloned in pLNH-ST.

pLNH-ST differs from other traditional 5S rDNA-based hcn yeast integrating vectors in that it also contains a functional yeast ARS sequence (Struhl et al., 1979;
30   Stinchcomb et al., 1980; Chan and Tye, 1980) as shown in Figure 9. Thus, pLNH-ST is both a replicative vector and an integrative vector. Uniquely, pLNH-ST functions first

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as a replicative vector then as an integrative vector in
the development of recombinant yeasts containing high
copies of integrated XR, XD, and XK. The ARS fragment
was inserted at the EcoR1 site of pUCKm 6. In addition,
5   pLNH-ST also contains the kanamycin resistance gene (Km^R)
and the ampicillin resistance gene (Ap^R). Km^R functions
as a geneticin resistance gene in yeasts and will confer
its yeast transformants resistant to geneticin. The XhoI
site of Km^R was removed by PCR technique without
10   affecting its activity. Both Km^R and Ap^R are part of the
original pUCKm 6 plasmid.

As noted above, the above-described vectors differ
from those used in state-of-the-art techniques by
15   containing an ARS sequence. In addition, in prior-
reported methods for making hcn yeast integrants,
integration of the cloned genes has taken place
instantly, at the moment when the yeast cells are
transformed with the exogenous genes. To the contrary,
20   in accordance with preferred modes of the invention,
integration of the cloned genes continues to take place
gradually, long after transformation has been completed.
In particular, transformation is established first via
the presence of replicative plasmid, such as pLNH-ST, in
25   the transformed yeast cells, and integration takes place
only gradually via repeated replication of the
transformants on plates containing selective medium.

Thus, this invention relates the use of the
30   following procedures to develop yeast or other cell
transformants containing hcn integrated cloned gene(s).

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Host cells which contain reiterated DNA sequences, for
example yeast or eukaryotic cells, are transformed with a
replicative/integrative plasmid, such as pLNH-ST, and
transformants containing high-copy numbers of the
5   replicative/integrative plasmid are selected. The
resulting selected transformants are repeatedly
replicated onto fresh selective plates and grown to high
cell density for a sufficient number of times to
integrate the desired number of copies of the exogenous
10   DNA, followed by culturing the transformants in non-
selective medium for a sufficient number of generations
to remove the replicative/integrative plasmids from the
transformants. The resulting transformants can then be
cultured in selective medium, and those transformants
15   retaining their capability to effectively grow in
selective medium will be those that contain hcn of the
desired exogenous genes integrated into the chromosome of
the yeast or other host cells. For example, fusion 1400
yeast has been transformed with pLNH-ST according to the
20   above described procedures, and the resulting stable
recombinant yeast, 1400(LNH-ST), can coferment both
glucose and xylose better than 1400(pLNH 32) and
1400(pLNH33), as shown in Figure 11. Importantly, the
newly-developed stable recombinant yeast, 1400(LNH-ST),
25   can still ferment both glucose and xylose with equal
efficiencies after being cultured in non-selective medium
for 4, 20, and 40 generations as shown in Figure 7, while
1400(pLNH 32) and 1400(pLNH33) will lose most of their
activity for fermenting xylose after 20 generations of
30   being cultured in non-selective medium (Figures 5 and 6).
Furthermore, 1400(LNH-ST) has subsequently been cultured
in non-selective medium for several hundred generations,

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and still retains its full activity in cofermenting both
glucose and xylose.

In the preferred methods for developing stable hcn
5   integrants, a common selection marker(s) is/are used for
the selection and maintenance of both the plasmid-
mediated activity and the activity contributed by the
integrated genes with the same selective medium. In the
present work, the common selection markers are the three
10 cloned xylose metabolism genes, XR, XD, and XK, and the
common selective medium is either rich or minimal medium
(for yeasts) containing xylose. In addition, these
cloned genes serve as the selection markers in rich
medium for most *Saccharomyces* yeasts, since the
15 applicants have shown that most of the *Saccharomyces*
yeasts do require the presence of a carbon source, such
as xylose, for growth even in rich medium (Figure 8).
Although it is not crucial for yeasts chosen as hosts to
require the presence of a carbon source in rich medium
20 for growth, it is, nevertheless, much more convenient to
be able to select the desired integrants on plates
containing rich medium with xylose rather than on plates
containing minimal medium with xylose. Preferred hosts
for transformation in the present invention belong to the
25 *Saccharomyces* species, since they are usually
extraordinarily effective for fermenting glucose. In the
event that species of yeasts desired for use as hosts for
integrating high copy numbers of xylose metabolizing
genes are found not to require the presence of a carbon
30 source for growth in rich medium, a suitable mutant of
that species which does require the carbon source in rich
medium can be isolated using conventional procedures.

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The replicative/integrative plasmid, such as pLNH-
ST, for achieving hcn integration also desirably contains
a second selection means for the selection of replicative
5   plasmid-mediated transformants. For pLNH-ST, the second
selection mechanism utilizes both Km^R and Ap^R as
selectable markers. Although it is not crucial for a
replicative/integrative vector to contain a second
selection system, it will provide more preferred vectors,
10   particularly if the ARS vector is not sufficiently stable
even in the presence of the selection pressure, and the
transformants have the tendency to lose most of their
plasmids prior to integrating sufficient copies of the
desired genes. When using vectors which contain a second
15   selection mechanism, the transformants may be cultured in
the presence of the second selective reagent to boost
their plasmids' copy number, or to re-transform the
transformants with the same vector but using the second
selection mechanism to re-select the transformants so
20   that the integration process can be continued or re-
initiated.

The use of both Km^R and Ap^R as the second selection
system is desirable for the applicants' preferred yeast.
25   Km^R can be a dominant selection marker for transforming
yeasts that are resistant to geneticin, but some yeasts
are naturally resistant to geneticin without acquiring
the plasmid containing Km^R. As a result, Km^R alone is
not a preferred selection marker for the selection of
30   yeast transformants. On the other hand, Ap^R can be
effectively expressed in most yeasts, but it generally

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cannot be used as a dominant selection marker for yeast
transformation because most yeasts are naturally
resistant to ampicillin. However, both Km^R and Ap^R
together serve as an excellent dominant selection system

5   for most yeasts, particularly the *Saccharomyces* yeasts.
To use such a selection system, the transformants are
first selected on plates containing YEPD (1% yeast
extract, 2% peptone, 2% glucose) and proper
concentrations of geneticin (20-80 µ g/ml, varying from

10   species to species). The resulting transformants are
screened for the expression of the Ap^R by the
penicillinase test (Chevallier and Aigle, 1979) to
identify true transformants.

15   The presence of Ap^R in pLNH-ST (Figure 9) and
related replicative/integrative plasmids also serves
another function. Since Ap^R is only present in the
replicative plasmid and not present on the fragment
integrated into the yeast chromosome, the ampicillin test

20   also serves as a convenient process for identifying those
transformants containing hcn integrated cloned genes but
not plasmid vectors.

A feature of the inventive approach for providing

25   stable recombinant yeasts containing hcn integrated
gene(s) is that the number of copies of the gene(s) to be
integrated can easily be controlled. For example, more
copies of the XR-XD-XK genes can be inserted into the
fusion yeast 1400 chromosome if another selection marker,

30   such as Km^R, is inserted into the 5S rDNA fragment (or
the targeting sequence). Furthermore, the inventive

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methods for the development of hcn yeast integrants are
also easier to accomplish than other reported approaches,
wherein experimental conditions may have to be adjusted
and controlled and the transformation process may have to
5 be repeated before a stable strain could be obtained.

Thus, the applicants have improved upon the
stability of prior recombinant xylose-fermenting yeasts,
such as 1400 (pLNH32) and 1400 (pLNH33), and developed
10 advantageously stable recombinant yeasts, for example
1400(LNH-ST), that will not require the presence of
selection pressure to maintain the cloned genes and are
also as effective as or even more effective for
cofermenting glucose and xylose than 1400(pLNH32) and
15 1400(pLNH33). Furthermore, the applicants have also
developed a convenient method that has provided the
facile hcn integration of exogenous gene(s) into the
cellular chromosome, wherein the number of copies of the
gene(s) to be integrated is also readily controllable.
20

Similar to 1400 (pLNH32) and 1400(pLNH33), the
preferred stable genetically engineered xylose-fermenting
yeasts of the invention can also effectively coferment
both glucose and xylose. This is because the XR, XD, and
25 XK genes inserted into the chromosome of the new yeast
hosts are all fused to intact 5' non-coding sequences
from genes that can be efficiently expressed in yeast,
encoding the production of high levels of enzymes, and
also which are not inhibited by the presence of glucose
30 in the medium. For example, the intact 5' non-coding DNA
sequences that contain all the genetic elements for
efficient expression of the glycolytic genes and for the

- 25 -

production of high levels of glycolytic enzymes are suitable as replacements for the intact 5' non-coding sequences of XR, XD, and XK for these purposes.

5   The XR, XD, and XK cloned on pLNH-ST are from *Pichia stipitis* (XR and XD) and *Saccharomyces cerevisiae*. (XK). However they can be from any microorganisms as long as they can produce high levels of the respective enzymes after they have been fused to

10   the proper 5' non-coding sequences containing effective promoters, ribosomal binding sites, etc. For example, these three genes are well known to occur in a wide variety of microorganisms and numerous XR, XD and XK genes have been identified and isolated. The particular

15   source of these genes is thus not critical to the broad aspects of this invention; rather, any DNAs encoding proteins (enzymes) having xylose reductase activity (the ability to convert D-xylose to xylitol with NADPH and/or NADH as cofactor), xylitol dehydrogenase activity (the

20   ability to convert xylitol to D-xylulose with NAD$^+$ and/or NADP$^+$ as cofactor), or xylulokinase activity (the ability to convert D-xylulose to D-xylulose-5-phosphate) will be suitable. These genes may be obtained as naturally-occurring genes, or may be modified, for

25   example, by the addition, substitution or deletion of bases to or of the naturally-occurring gene, so long as the encoded protein still has XR, XD or XK activity. Similarly, the genes or portions thereof may be synthetically produced by known techniques, again so

30   long as the resulting DNA encodes a protein exhibiting the desired XR, XD or XK activity.

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As examples, suitable sources of XR and XD genes
include xylose-utilizing yeasts such as *Candida
shehatae, Pichia stipitis, Pachysolen tannophilus*,
5   suitable sources of XK genes include the above-noted
xylose-utilizing yeasts, as well a xylose non-utilizing
yeasts such as those from the genus *Saccharomyces*, e.g.
S. cerevisiae, the genus *Schizosaccharomyces*, e.g.
Schizosaccharomyces pombe, and bacteria such as
10 *Escherichia coli*, Bacillus species, *Streptomyces*
species, etc. Genes of interest can be recovered from
these sources utilizing conventional methodologies. For
example, hybridization, complementation or PCR
techniques can be employed for this purpose.
15

A wide variety of promoters will be suitable for
use in the invention. Broadly speaking, yeast-
compatible promoters capable of controlling
transcription of the XR, XD or XK genes will be used.
20 Such promoters are available from numerous known
sources, including yeasts, bacteria, and other cell
sources. Preferably, the promoters used in the
invention will be efficient, non-glucose-inhibited
promoters, which do not require xylose for induction.
25 In this regard, an "efficient" promoter as used herein
refers to a 5' flanking sequence which provides a high
level of expression of the fused gene. Promoters having
these characteristics are also widely available, and
their use in the present invention, given the teachings
30 herein, will be within the purview of the ordinarily
skilled artisan, as will be the fusion of the promoters
to the XR, XD and XK genes, the cloning of the

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promoter/gene fusion products into appropriate vectors
and the use of the vectors to transform yeast. All of
these manipulations can be performed using conventional
genetic engineering techniques well known to the art and
5   literature.

The yeast DNA replication origin, e.g. the ARS
containing DNA fragment, can be obtained from yeast
chromosomal DNA or from chromosomal DNA of other
10 organisms, so long as the DNA fragment can function as an
active replication origin to support autonomous
replication of plasmid in the host chosen for hcn
integration. DNA fragments which function as ARSs can
readily be isolated by incorporating randomly-digested
15 DNA fragments into an *E. coli* plasmid, followed by
transformation of the desired host organism, e.g. a
Saccharomyces yeast, with the resulting bank of plasmids,
as reported in the literature (Stinchcomb et al., 1980;
Ho et al., 1984).
20

Novel methods have been used to create the stable
strains of the present invention. Nevertheless, there
are several lines of evidence indicating that the cloned
genes are not on a replicative plasmid and have been
25 integrated into the host genome. For example,
chromosomal DNA isolated from 1400(LNH-ST) can be used as
template for the isolation of the cloned genes, including
the fusions containing both the 5s rDNA and the cloned
gene sequences, by the polymerase chain reaction (PCR).
30 Also, while few plasmids (pLNH-ST) can be recovered from
1400(LNH-ST) via transformation of *E. coli* (Ward, 1990),
under the same conditions, hundreds of pLNH32 or pLNH33

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plasmids can be recovered from 1400 (pLNH32) and
1400(pLNH33), respectively. Furthermore, the initial
1400 fusion yeast transformants containing high copy
numbers of the replicative plasmid pLNH-ST are unstable
5   (with respect to their capability to ferment xylose) but
positive for penicillinase (enzyme encoded by Ap^R) test
(Chevallier and Aigle, 1979). On the contrary, the final
stable transformants, 1400(LNH-ST), which retain their
capability for fermenting xylose without the presence of
10   selection, are found to be negative for penicillinase
test. This is expected if the exogenous DNA is
integrated at the site of 5S rDNA since Ap^R is not part
of the DNA fragment to be integrated into the host
chromosome. It is also possible that some of the stable
15   yeast transformants may contain exogenous genes
integrated at the ARS sites of the yeast chromosome.

        For purposes of promoting a further understanding of
the present invention and its features and advantages,
20   the following Examples are provided. It will be
understood, however, that these Examples are
illustrative, and not limiting, of the invention.

25   EXAMPLE 1
              SYNTHESIS OF THE 5S rDNA FRAGMENT BY PCR

        For the synthesis of the 5S rDNA fragment by PCR (to
serve as the yeast DNA sequence for targeting high-copy-
30   number integration into the yeast chromosome), the
following oligonucleotides were synthesized and used as
the primers for PCR reactions according to the published
5S rDNA sequence (Valenzuela et al., 1977). In addition

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to the 5S rDNA sequence, additional nucleotides
specifying the Sal I restriction site were also added to
the 5' terminal of primers to facilitate the cloning of
the PCR synthesized 5S rDNA into an *E. coli* plasmid.

5

Oligonucleotide I: TTAGTCGACGTCCCTCCAAATGTAAAATGG.

Oligonocleotide II: AATGTCGACGTAGAAGAGAGGGAAATGGAG

10   Chromosomal DNA isolated from fusion yeast 1400 was
used as the template for the PCR reaction. The PCR
synthesized 5S rDNA fragment was first cloned into the
E.coli pBluescript II KS(-) plasmid (Stratagene Cloning
Systems, La Jolla, CA) at its SalI site. The resulting
15 plasmid was designated as pKS-rDNA(5S).

EXAMPLE 2
INSERTION OF XHOI SITE INTO CLONED 5S rDNA SEQUENCE

20   The nucleotide sequence between -29 and -56 of the
5S rDNA sequence (Valenzuela et al., 1977) was modified
by oligonucleotide-mediated site-specific mutagenesis
(Kunkel, 1985; Kunkel et al., 1987).   As a result, an
XhoI restriction site was inserted at the specific site
25 described above. The protocol provided by Bio-Rad
Laboratories, Inc. for oligonucleotide-mediated site-
specific mutagenesis was followed to accomplish this
task, except that pKS plasmid was used rather than
plasmid pTZ18U or pTZ19U. The resulting plasmid
30 containing the mutated 5S rDNA was designated as pKS-5S
rDNA(XhoI). The following oligonucleotide was used to

- 30 -

carry out the site-specific mutagenesis:
GAGGGCAGGCTCGAGACATGTTCAGTAGG.

EXAMPLE 3

ISOLATION OF DNA FRAGMENTS FROM *S. CEREVISIAE*
DNA OR OTHER DNA FUNCTIONING AS ARS IN YEASTS

5

S. cerevisiae DNA (or DNA from other yeasts or other
organisms) was digested with Sau3A restriction enzyme and
10 cloned into the Bam H1 site of pUCKm6 (Figure 12) (Ho, et
al., 1984). The resulting bank of plasmids was used to
transform *S. cerevisiae*. Those transformants that were
capable of growing on plates containing YEPD (1% yeast
extract, 2% peptone, and 2% glucose) and 50 µg/ml
15 geneticin and which were also positive for the
penicillinase test (Chevallier and Algle, 1979) were
selected. The plasmids from the selected true
transformants were recovered by a procedure similar to
that described by Ward (1990).
20

The yeast DNA fragments inserted in pUCKm6 (Figure
12) and recovered from the yeast transformants should all
contain a segment of DNA that can function as an ARS
(autonomous replicating sequence) in *S. cerevisiae*,
25 possibly in other yeasts as well. The DNA inserts were
digested with various restriction enzymes and the
resulting DNA fragments were re-inserted into pUCKm6.
The latter plasmids were used to retransform *S.
cerevisiae*. Any properly-sized restriction fragments
30 that can make pUCKm6 function effectively as a yeast
plasmid must contain an effective "ARS" and can be used

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to construct replicative/integrative vectors such as
pLNH-ST for high-copy-number integration of exogenous
gene(s) into the chromosomes of *S. cerevisiae*. These
restriction fragments are also likely to function as
5   ARS's in other yeasts, and are suitable for the
construction of replicative/integrative plasmids for
other yeasts.

EXAMPLE 4
10   REMOVAL OF THE XhoI RESTRICITON SITE FROM THE
GENETICIN (KANAMYCIN) RESISTANCE GENE, KmR

The geneticin (kanamycin) resistance gene, Km^R, from
Tn 903 (A. Oka et al., 1981) and the 5S rDNA fragment
15   described in Example 1 are part of the plasmid designed
for the integration of multiple copies of exogenous genes
into the yeast chromosome. However, Km^R contains an XhoI
site in its coding sequence. This is in conflict with
the fact that an XhoI site has been engineered into the
20   center of the cloned 5S rDNA sequence to be used for
inserting exogenous genes such as XR, XD, and XK into the
plasmid for integration. Thus, it is necessary to remove
the XhoI site from Km^R. This can be accomplished by a
number of different approaches. The applicants chose to
25   use site-specific mutagenesis by the overlap extension
PCR technique (S. N. Ho, et al., 1989) to remove the XhoI
site from Km^R without changing its amino acid coding
sequence and without affecting the catalytic activity of
the enzyme encoded by the gene. The Km^R gene cloned in
30   pUCKm6 (Figure 12) was converted to Km^R(-Xho) as
described above.

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The four oligonucleotides used to accomplish this
task are listed below.

Oligonucleotide I: GGCCAGTGAATTCTCGAGCAGTTGGTG

5

Oligonucleotide II: TGGAATTTAATCGCGGCCCCTAGCAAGACG

Oligonucleotide III: TTACGCCAAGCTTGGCTGC

10   Oligonucleotide IV: TTCAACGGGAAACGTCTTGCTAGGGGCCGC

pUCKm6 (Figure 12) is a derivative of pUC9. Part of
Oligo I and the entire Oligo III are synthesized
according to the sequence of the polylinker region of
15   pUC9 (Sambrook, et al. 1989).

The above-described genetic manipulation of pUCKm6
not only resulted in the deletion of the XhoI restriction
site from the coding region of Km^R but also inserted an
20   XhoI restriction site between the Km^R coding sequence and
the EcoRI site of pUCKm6. The resulting plasmid was
designated as pUCKm(-XhoI)(+XhoI). The addition of an
XhoI site downstream to the Km^R coding sequence is to
facilitate the insertion of the 5S rDNA fragment
25   described in Example 1 into the newly developed plasmid
pUCKm(-Xho)(+Xho).

EXAMPLE 5
CONSTRUCTION OF PLASMID pLNH-ST
30

The plasmid pUCKm(-XhoI)(+XhoI) described in Example
4 was used for the construction of pLNH-ST, shown in

Figure 9. First, the Sal I fragment containing the 5S
rDNA(XhoI) was isolated from pKS-5S rDNA(XhoI) and
inserted at the XhoI site of
pUCKm(-XhoI)(+XhoI). The resulting plasmid was

5   designated as pUCKm-rDNA(5S). To the latter plasmid, an
EcoRI fragment containing an effective ARS isolated from
S. cerevisiae (according to the procedure described in
Example 3) was inserted into the EcoRI site of pUCKm-5S
rDNA, and the resulting plasmid was designated as pUCKm-

10   5S rDNA-ARS. To the latter plasmid, the XhoI fragment
from pKS(-)-KK-AR-KD-3 containing the cloned XR, XD, and
XK fused to yeast alcohol dehydrogenase promoter (XR),
and pyruvate kinase promoter (for both XD & XK), were
inserted into the XhoI site located at the center of the

15   cloned 5S rDNA sequence. The resulting plasmid, pUCKm-
rDNA(5S)(KDR)-ARS, also designated pLNH-ST, shown in
Figure 9.

EXAMPLE 6
TRANSFORMATION OF FUSION YEAST 1400 WITH pLNH-ST AND SELECTION OF
STABLE TRANSFORMANTS 1400 (LNH-ST)

20

pLNH-ST was used to transform fusion strain 1400 by
electroporation under the conditions used for

25   transformation of strain 1400 by plasmids pLNH32 and
pLNH33 (International Publication No. 95/13362, May 18,
1995, publishing International Application No.
PCT/US94/12861, filed November 8, 1994). Briefly, fifty
ml yeast cells, grown to early log phase (Klett Unit (KU)

30   140-190), were centrifuged to remove the medium, washed
twice with cold water, once with cold 1 M sorbitol, and
resuspended in 200 μl 1 M sorbitol. Sixty μl of the
cells were transferred into a 4 ml presterilized plastic

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tube (with cap) and to which 1 μg plasmid DNA was added.
Fifty μl of the resulting cells and plasmid mixture were
pipetted into a precooled gene pulser cuvette with a 0.2
cm electrode gap and the content in the cuvette was
5   subjected to pulse by the gene pulser with a pulse
controller (BioRad) at 2.0 KV, 25 μF, 200 ohms.

Immediately, .50 ml YEPD was added to the cuvette.
The content of the cuvette was transferred to a new 4 ml
10   sterilized plastic tube and incubated at 30°C for 1 hr.
100 μl of the cells were plated on agar plates containing
YEPD and 40 μg/ml G418 (geneticin). Fast growing
colonies were selected and replicated on another plate
containing the same medium. The selected colonies were
15   subjected to the ampicillin test (Chevallier and Aigle,
1979) until a positive one was identified. The above-
described electroporation procedure is based on that
reported by Becker and Guarente (1971).

20   Once a transformant had been positively identified
by the penicillinase test, it was maintained on a YEPX
(1% yeast extract, 2% peptone, 2% xylose) plate.
Initially, the transformants were very unstable. They
lost their xylose fermenting capability if cultured in
25   YEPD medium over 20 generations. However, by continuing
to culture the transformants to stationary phase on YEPX
plates, and repeatedly transferring them to fresh YEPX
plates, the transformants gradually became stable with
regard to their capability to ferment xylose. Once
30   stable, the transformants could be cultured in non-
selective medium for several hundred or more generations

- 35 -

and were still capable of co-fermenting both glucose and
xylose, as demonstrated in Example 8.

EXAMPLE 7
Co-FERMENTATION OF GLUCOSE AND XYLOSE WITH 1400 (LNH-ST)

A mixture of glucose and xylose (approximately 10%
glucose and 5% xylose) was fermented by strain 1400 (LNH-
ST) under the conditions described below. The seed
cultures of 1400 and 1400 (LNH-ST) were cultured
aerobically in liquid YEPD medium until mid-log phase
(between 400-450 Klett Units (KU)) and stored at 4°C.
New seed cultures were prepared once a month by
transferring 2 ml of the culture to 50 ml of fresh YEPD
and cultured as described above. 2 ml of the seed
cultures of 1400 (LNH-ST) were inoculated into 100 ml of
YEPD medium in a 300 ml Erlenmeyer flask equipped with a
side-arm which allowed direct monitoring of the growth of
the yeast cultures by the Klett colorimeter. The culture
was incubated in a shaker at 30°C and 200 rpm
aerobically.

When the cell density reached mid-log phase (400-450
KU), 20 ml (50%) glucose and 10 ml (50%) xylose were
added to the flask. After thorough mixing, 1 ml of the
culture mixture was removed from the flask to serve as
the zero sample. The flask was then sealed with Saran
wrap to allow fermentation to be carried out
anaerobically. One ml samples of the fermentation broth
were removed at proper intervals (every 6 hrs.) to serve
as samples for measuring glucose, xylose, xylitol, and
glycerol contents of the broth during fermentation by

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HPLC as described in Example 9. The results, shown in
Figure 11 , demonstrate that the genetically engineered
yeast 1400 (LNH-ST) can co-ferment most of the 10%
glucose and 5% xylose to ethanol in 30 hrs. The

5   fermentation was carried out under normal conditions,
without requiring special medium or pH, and also without
requiring growth of yeast to high cell density. Thus,
the genetically engineered 1400 (LNH-ST) can effectively
co-ferment high concentrations of both glucose and xylose

10 to ethanol with very little xylitol produced as a by-
product. In comparison to the recombinant *Saccharomyces*
1400 (pLNH32) and 1400 (pLNH33) shown in Figures 2 and 3,
1400 (LNH-ST) co-fermented both glucose and xylose
somewhat better than the two previously developed yeasts.

15

EXAMPLE 8
COMPARISON OF THE STABLE STRAIN 1400 (LNH-ST) WITH 1400 (LNH32)
AND 1400 (LNH33) IN CO-FERMENTING GLUCOSE AND XYLOSE AFTER CULTURE

20 IN NON-SELECTIVE MEDIUM FOR 4, 20, AND 40 GENERATIONS.

As described in Example 7, 2 ml each of the seed
cultures of 1400 (LNH-ST), 1400 (LNH32), and 1400 (LNH33)
were inoculated into 50 ml YEPD in separate 250 ml

25 Erlenmeyer flasks equipped with side-arms. After the
cells were cultured to 400-450 KU, 2 ml of the fresh
culture from each flask were transferred to a new flask.
This process was repeated 10 times for 1400 (LNH-ST) and
5 times for 1400 (LNH32) and 1400 (LNH33). The 1400

30 (LNH-ST) cultures that were cultured for 4, 20, and 40
generations in non-selective medium (each transfer being
considered as four generations cultured in non-selective
medium) were used to co-ferment glucose and xylose under

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similar conditions described in Example 7. The fermentation samples were taken and analyzed identically as described in Example 7. Similarly, the 1400 (LNH32) and 1400 (LNH33) cultures that were cultured for 4 and 20

5   generations in non-selective medium were used to co-ferment glucose and xylose. Samples were again taken at proper intervals after fermentation was initiated for analysis by HPLC and compared in Figures 4 to 6. These results clearly demonstrate that 1400 (LNH-ST) is far

10   more stable than 1400 (LNH32) and 1400 (LNH33) in maintaining its xylose fermenting capability after being cultured in non-selective medium for more than 40 generations.

15   **EXAMPLE 9**
              HPLC ANALYSIS OF FERMENTATION SAMPLES

        The samples containing the fermentation broth (0.6 ml to 1.0 ml) removed from the cultures were kept in 1.5

20   ml Eppendorf tubes. The cells and other residues were removed by centrifugation in microfuge (topspeed) for 10 min. The supernatant was diluted 10 fold. The resulting diluted samples were analyzed for its ethanol, glucose, xylose, xylitol, and glycerol contents by high

25   performance liquid chromatography (HPLC), using a Hitachi system according to the following conditions.

    · Column: BioRad HPX-87H

    · Mobile Phase: 0.005 M H_2SO_4

    · Flow Rate: 0.8 ml/min.

30   · Detection: RI detector

    · Temperature: 60°C

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· Injection Volume: 20 μl

EXAMPLE 10
GENETIC CHARACTERIZATION OF CHROMOSOMAL DNA FROM
THE STABLE TRANSFORMANTS 1400 (LNH-ST)

5

Based on the restriction and PCR analysis, the
genetic map (the order and orientation) of the cloned

10   genes, KK, AR, KD, and 5S rDNA present in pLNH-ST, have
been determined as shown in Figure 9B. Experiments have
been designed to determine whether these genes (KK, AR,
and KD) have been integrated into the loci of the 5S
rDNA. If these genes have been integrated into the yeast

15   chromosome at the loci of the 5S rDNA as anticipated, the
correct size of DNA fragments containing the following
combination of partial or intact genes such as 5S rDNA-
KK; 5S rDNA-KD; KK-AR, and AR-KD should have been
obtained by using 1400 (LNH-ST) chromosomal DNA as the

20   template and the oligonucleotides indicated on the
genetic map (Figure 9B) as the primers to carry out DNA
synthesis by PCR. If these genes have not been
integrated into the yeast chromosome, no such combination
of genes or gene fragments should have been obtained by

25   the above described experiments. If these genes have
been integrated elsewhere in the yeast chromosome rather
than at the loci of 5S rDNA, some of the above described
combination of genes or gene fragments should be obtained
from the above described experiments, but not those

30   containing the 5S rDNA fragment; such as 5S rDNA-KK and
5S rDNA-KD. For carrying out the above described
experiments, chromosomal DNA was isolated from 1400 (LNH-
ST), using the protocol provided by Qiagen, Chatsworth,

CA.   Positive results were obtained from PCR synthesis by
using the following pairs of primers (see Figure 9):
Oligo 25 and Oligo 369; Oligo 26 and Oligo 369; Oligo 370
and Oligo 96; Oligo 97 and Oligo 99; Oligo 982 and
5   Oligo27. Thus, based on these analyses, the DNA fragment
containing KK-AR-KD seems indeed being integrated in the
1400 yeast chromosome at its 5S rDNA loci.

- 40 -

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level of skill possessed by those in the art and are each
5 hereby incorporated by reference as if individually
incorporated by reference and fully set forth.

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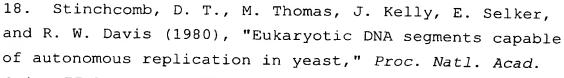30   "Multiple, Tandem Plasmid Integration in *Saccharomyces*

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